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Formation of Mutagens Following Chlorination of Humic Acid: A Model for Mutagen Formation during Drinking Water Treatment

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16. ABSTRACT
Aqueous chlorination of humic acids results in the formation of compounds with direct-acting mutagenic activity in the Ames/Salmonella plate assay for tester strains TA98, TA100, TA1535, TA1537, and TA1538. The addition of a rat liver microsomal fraction (S-9) plus cofactors causes a substantial decrease of activity, the extent of which is tester strain dependent. The non-chlorinated humic acids are not mutagenic either in the presence or absence of S-9. Formation of mutagenic activity and of total organic halogen (TOX) is linearly related to humic concentration in the range of 0.2-1.6 mg/ml total organic carbon (TOC), and to chlorine concentration in the range of 0.1-1.0 chlorine equivalents per mole of carbon. Mutagenic activity is also detectable, after sample concentration by lyophilization, upon chlorination at a humic acid level of 0.2 mg/ml TOC. The mutagenic activity is due predominantly to non-volatile compounds. Production of mutagens is greatly dependent on the chlorination pH, with a pattern of decreasing mutagenic activity with increasing pH. This order of activity could be at least partially explaiend by the alkali-lability of the compounds. Chlorination of commercial humic acids is proposed as a model for examinationonf mutagen formation during water chlorination.

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Formation of mutagens following chlorination of humic acid
A model for mutagen formation during drinking water treatment

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Summary

Aqueous chlorination of humic acids results in the formation of compounds with direct-acting mutagenic activity in the Ames/Salmonella plate assay for tester strains TA98, TA100, TA1535, TA1537 and TA1538. The addition of a rat-liver microsomal fraction (S9) plus cofactors causes a substantial decrease of activity, the extent of which is tester strain dependent. The non-chlorinated humic acids are not mutagenic either in the presence or absence of S9. Formation of mutagenic activity and of total organic halogen (TOX) is linearly related to humic concentration in the range of 0.2–1.6 mg/ml total organic carbon (TOC), and to chlorine concentration in the range of 0.1–1.0 chlorine equivalents per mole of carbon. The mutagenic activity is due predominantly to non-volatile compounds. Mutagenic activity is also detectable, after sample concentration by lyophilization, upon chlorination at a humic acid level of 0.02 mg/ml TOC. The specific mutagenic activities (per mg TOX), and also the degree of chlorine incorporation into humic acid, at 0.02 mg/ml TOC are similar to those present after chlorination at 1 mg/ml TOC. Production of mutagens is greatly dependent on the chlorination pH, with a pattern of decreasing mutagenic activity with increasing pH. This order of activity can be at least partially explained by the alkali lability of the compounds. Chlorination of commercial humic acids is proposed as a model for examination of mutagen formation during water chlorination.

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Naturally occurring humic and fulvic acids account for the bulk of the organic matter which is present in most surface waters. Considerable attention was given to natural aquatic humic substances following the discovery by Rook in 1974 that the presence of trihalomethanes in drinking water was due to the reaction of chlorine with 'precursor' substances, which were postulated to be humic materials. Rook (1976) and Stevens et al. (1978) subsequently confirmed that chlorination of humic or fulvic acids results in trihalomethane formation. In addition to the trihalomethanes, a variety of both chlorinated and non-chlorinated organic products have since been shown to be formed as a result of aqueous chlorination of humic materials (Christman et al., 1980; Coleman et al., 1983; Rook, 1977). The identification of many of these products in drinking water (Rook, 1980; Coleman et al., 1983) suggests that humic materials might serve as precursors to many of the organic chemicals which are formed during the chlorination stage in drinking water treatment.

The organic compounds that have thus far been identified in drinking water are mostly volatile organics. As a whole the volatile organics (i.e. those detected by gas chromatography without derivatization) represent only 5–10% of the total organic content of drinking water (National Academy of Sciences, NRC report, 1977), and may account for only a minor portion of the halogenated compounds present (Glaze et al., 1979; Rook, 1980). Little is currently known about the identity of the non-volatile organic compounds in drinking water mainly because they have proven to be difficult to identify using current analytical techniques. As a result the health risks which are posed by the presence of these compounds remains largely unknown.

Concern over potential human health hazards has been heightened by the widespread recognition in recent years of mutagenic activity exhibited by these uncharacterized organics (Loper et al., 1978; Glatz et al., 1978; Nestmann et al., 1979; Forster et al., 1981; Heartlein et al., 1981; Kool et al., 1981). The presence of non-volatile mutagens in drinking water has been directly linked with water chlorination practices (Cheh et al., 1980; Maruoka and Yamanaka, 1980; de Greef et al., 1980). It seems likely that these mutagenic by-products of chlorination are also formed as a result of the reaction of chlorine with humic materials present in the source waters. Direct support for this hypothesis has been provided by recent findings from this laboratory which demonstrate that both humic and fulvic acids isolated from natural lakes, as well as a commercially obtained humic material, react with chlorine to form direct-acting mutagenic products (Bull et al., 1982). The present report provides more detailed findings on the reaction conditions necessary for the formation of these mutagenic compounds, and preliminary results on the characteristics of the mutagens. Part of this work has been presented in abstract form (Meier et al., 1982).

Materials and methods

Preparation and chlorination of humic samples

The method for preparation of humic samples was modified slightly from that

previously described (Bull et al., 1982). Humic acid (Fluka, Switzerland) was added to distilled water at a level of 4 g/l. The pH was adjusted to 7.0 (for neutral pH chlorination) or 11.5 (for high pH chlorination) by the addition of NaOH. The solution was then stirred with mechanical stirring for 18–24 h in order to dissolve most of the humic material. The solution was then centrifuged at $16000 \times g$ for 20 min, decanted, and recentrifuged. The supernatant, representing soluble humic material, was then diluted to give a final total organic carbon content of approximately 2.3 g/l.

Chlorination of the humic sample was carried out using a solution of chlorine in distilled water. For chlorination at neutral pH, the chlorine solution was prepared at a concentration of 10–12 g/l by bubbling chlorine gas into a solution of 1.1% NaOH in water until a pH of 7.5 ± 0.3 was reached. For chlorination at high pH, the chlorine gas was bubbled into a solution of 2.2% NaOH in water until a pH of 11.5 was reached. The concentration of chlorine was determined using the starch-iodine method (American Public Health Association, 1975). The chlorine solutions were used immediately after preparation. The reaction of chlorine with humic material was carried out in amber glass, teflon-capped bottles for 90 h, at which time chlorine consumption was greater than 99% complete. The effect of varying the chlorine concentration on the reaction was examined by maintaining a constant humic concentration at 1.0 g/l TOC and adding chlorine at different ratios of chlorine equivalents per mole of carbon. Alternatively, the effect of varying the humic concentration was examined by maintaining a constant 0.8 : 1.0 ratio of chlorine equivalents per mole of carbon and adding different amounts of the same humic stock solution. For chlorination under buffered conditions, potassium phosphate buffer was added to a final concentration of 0.25 M, followed by a readjustment of the pH to 7.0. Non-chlorinated samples were adjusted to pH 3 by the addition of HCl in order to maintain the sterility of the sample. Microbial contamination was not a problem after chlorination of the humic, even though there was usually no detectable chlorine residual. The samples were stored in teflon capped glass bottles at 4°C in the dark until time of assay.

For determining the effect of sample pH on stability of the mutagenic activity, a humic acid solution was prepared at 1 g/l TOC and chlorinated using a 1 : 1 ratio of chlorine equivalents per mole of carbon. A portion of this sample (pH 2.8) was saved and the rest was titrated to pH 7.0 using NaOH. After removing a portion, the remainder was titrated to pH 11.5 by further addition of NaOH. The samples were then stored at either 4°C or 23°C, and assayed for mutagenic activity at 1 h, 4 h, 24 h, 4 days and 7 days. At each of the assay times, the sample pH was checked and readjusted if necessary. Adjustment of pH utilized concentrated solutions of NaOH, thus keeping dilution of the samples to less than 5%.

Mutagenesis assays

Salmonella typhimurium his$^-$ strains TA1535, TA1537, TA1538, TA98 and TA100 were a gift of Dr. Bruce Ames (Univ. of California, Berkeley). Prior to use in assays these strains were checked for the presence of strain-specific markers (i.e. histidine dependence, UV sensitivity, crystal violet sensitivity, ampicillin resistance, sponta-

neous mutation rate and response to positive controls). 16–18 h cultures of the strains were used in assays. These cultures were inoculated from 'master plates' (Ames et al., 1981), and were grown in 2.5% Oxoid No. 2 broth (K.C. Biological) at 37°C with shaking. Assay for mutagenic reversion of the test bacteria to histidine independence was performed according to the standard plate method of Ames et al. (1975). Assays were carried out in duplicate, at 3 or 4 sample concentrations, both in the absence and presence of S9 (25 μl/plate). The S9 (9000 × *g* supernatant) liver homogenate fraction was obtained from Aroclor 1254 (Analabs, New Haven, CN) treated, male Sprague–Dawley rats according to the procedure used by Ames et al. (1975). Positive and negative (solvent) controls were performed with each assay. The positive controls in assay without S9 were: sodium azide (1 μg) for TA1535 and TA100, 2-nitrofluorene (2 μg) for TA98 and TA1538, and 9-aminoacridine (25 μg) for TA1537. For assays with S9, 2-aminoanthracene (1 μg) was used for all 5 strains. Distilled water was the solvent control in all cases.

The observation of a dose-related increase of 2-fold or higher above background was used as the criterion of a positive mutagenic response. Mutagenic activity was quantitated by least squares regression analysis of the linear portion of the dose–response curve. In this manner the slope (expressed as net revertants/ml of sample) ± the standard error of the slope was calculated and used for making comparisons of data.

Chemical analyses

The total organic carbon content of humic solutions was determined after appropriate dilution, using a Dohrmann Envirotech Model DC54 Organic Carbon Analyzer. Organic halogen content was determined by the GAC adsorption-microcoulometric method using a Dohrmann Total Organ Halogen Analyzer.

Results

Mutagenic responses of the 5 standard tester strains

Humic acid samples were evaluated for mutagenicity, before and after reaction with chlorine. Assays were carried out with the 5 Salmonella tester strains (i.e. *S. typhimurium* TA1535, TA1537, TA1538, TA98 and TA100) which have been recommended by Ames et al. (1975), and de Serres and Shelby (1979) for routine testing. The samples were prepared at an organic carbon concentration of 1 g/l and chlorination was performed at pH 7 using a 1 : 1 ratio of chlorine equivalents per mole of carbon. The results are presented in Figs. 1 and 2. Dose-related mutagenic activity was observed for each of the strains only after reaction of the humic substrate with chlorine. The mutagenic response for strain TA1535 (Fig. 1) was weak but slightly greater than 2-fold above background at the highest dose tested. Clear positive responses were apparent for the other 4 strains. The addition of a rat-liver S9 mixture resulted in decreased responses for all 5 strains, indicating the inactivation of direct-acting mutagens. The extent of decrease with a fixed amount of S9 was strain-dependent (compare TA100 vs. TA98, Fig. 2), suggesting that different com-

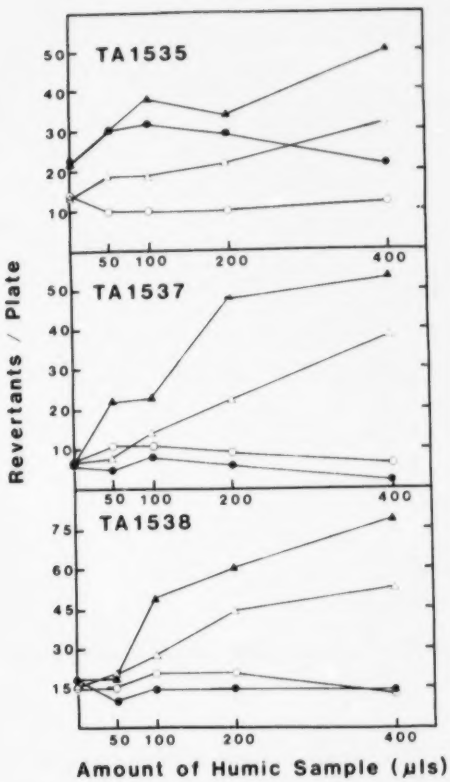

Fig. 1. Mutagenic responses of strains TA1535, TA1537, and TA1538 to humic acid, before and after chlorination. ●, non-chlorinated, −S9; ○, non-chlorinated +S9; ▲, chlorinated, −S9; △, chlorinated, +S9.

pounds are being detected with different degrees of inactivation. The addition of bovine serum albumin (BSA, crystalline grade, Sigma Chemical Co.) to the assay conducted with S9, in an amount equal to the protein content of the S9 (i.e., 0.75 mg/plate) did not affect the degree of inactivation; nor did substitution of BSA for the S9 result in any inactivation of the mutagens (data not shown).

Investigation of reaction condition requirements

In an effort to better understand the factors affecting the formation of the mutagenic compounds, the chlorination reaction conditions were more closely examined. Assays were performed only with *S. typhimurium* strains TA98 and TA100 since these 2 strains gave the highest responses and because they responded in a linear fashion with respect to dose, thus facilitating quantitative comparisons of

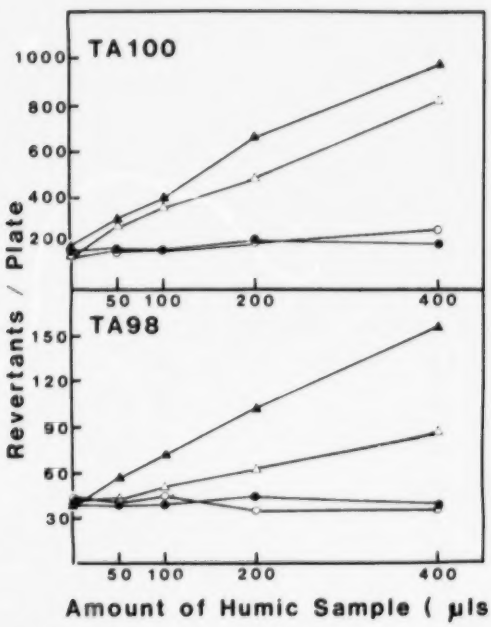

Fig. 2. Mutagenic responses of strains TA98 and TA100 to humic acid, before and after chlorination. ●, non-chlorinated, −S9; ○, non-chlorinated +S9; ▲, chlorinated, −S9; △, chlorinated, +S9.

data. Fig. 3 shows the effect of varying the humic concentration, while maintaining a constant chlorine : carbon ratio of 0.8 : 1.0, on mutagen formation and organic halogen formation.

The values for mutagenic activities were calculated from results of dose–response assays at each humic concentration. Mutagenic activity was related to humic concentration in an essentially linear manner over the range tested. Fig. 3 also shows that a good linear relationship existed between organic halogen formation and humic concentration (correlation coefficient, 0.9997). The level of chlorine incorporation into humic acid over this range was calculated to be 0.098 ± 0.01 moles of TOCl chlorine (measured as TOX) per mole of TOC carbon.

Mutagenic activity was barely detectable after chlorination at a humic acid organic carbon level of 0.16 mg/ml. Even this humic concentration is about 20–100 times higher than would be present in most raw surface waters used as drinking water sources, based on TOC levels present (Symons et al., 1975). Therefore, it seemed important to determine whether mutagen formation would occur upon chlorination at humic concentrations closer to those which might be encountered in drinking water treatment. In order to detect activity at these more realistic humic levels, concentration of the organics was attempted by lyophilization of the samples. This method has the advantage of being simple and relatively rapid, but has the

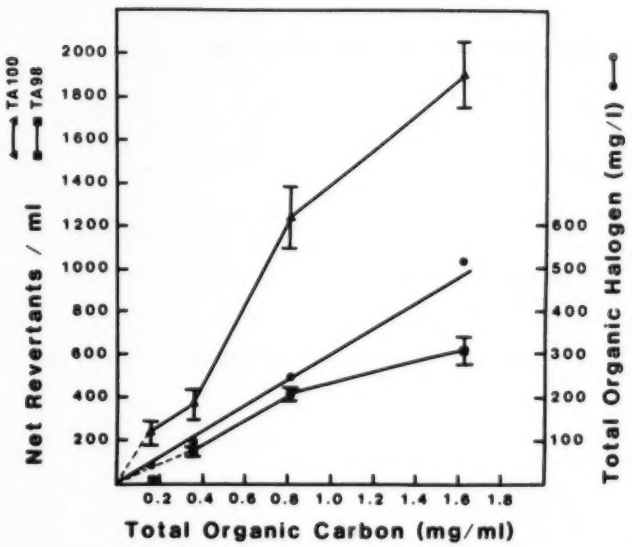

Fig. 3. Dependence of mutagen and organic halogen formation on humic concentration during chlorination. Mutagenicity values are slopes ± standard errors of dose–response curves at each humic concentration.

disadvantage that most volatile organics are lost in the process. The effect of lyophilization on recovery of mutagenic activity is shown in Table 1. In the case of the sample chlorinated at high humic concentration (i.e. 1 g/l), then lyophilized to dryness and reconstituted to its original volume (Sample B), nearly all of the mutagenic activity and greater than 80% of the TOX was recovered. This finding seemed to indicate that the mutagens were primarily non-volatile compounds. However, when a portion of this same sample was diluted 50-fold with distilled water, then lyophilized to dryness, and reconstituted to 1/50 of the initial volume (sample C), approximately half of the mutagenic activity and TOX was lost. The reason for this apparent concentration dependence of recovery efficiency is unclear. When chlorination was performed at a 45-fold lower TOC level (22 mg/l) followed by a 45-fold concentration using lyophilization (Sample D) mutagenic activity was still detectable. The levels of mutagenic activity and TOX were equivalent to about 45% of those observed for humic acid chlorinated at 1 g/l TOC, and then diluted 50-fold. Furthermore, when activity was calculated on the basis of organic halogen content of the samples after lyophilization, mutagen levels in the 22 mg/l TOC sample were nearly identical to the levels observed in the diluted 1 g/l TOC sample.

The volatile nature of the mutagenic compounds was further evaluated after purging the samples with helium for 2 h at room temperature to remove volatile organics. The results (Fig. 4) indicate that for both tester strains about 20% of the mutagenic activity is lost after purging.

TABLE 1

RECOVERY OF TOX AND MUTAGENIC ACTIVITY AFTER LYOPHILIZATION

Humic sample	Treatment	TOX [a] (mg/l)	Net revertants/ml ± S.E. [b]	
			TA98	TA100
(A) Chlorinated at 1 g/l TOC	Not lyophilized	391.0	395 ± 33	2386 ± 835
(B) Chlorinated at 1 g/l TOC	Lyophilized reconstituted to 1×	330.9(82)	343 ± 19(87)	2263 ± 103(95)
(C) Chlorinated at 1 g/l TOC; diluted to 0.02 g/l TOC	Lyoph., reconst. to 50×	204.7(51)	150 ± 13(38)	1201 ± 76(50)
(D) Chlorinated at 0.022 g/l TOC	Lyoph. reconst. to 45×	7.2 [c] 93.8 [d](23)	70 ± 15 (18)	534 ± 39 (22)
(E) Non-chlorinated; 0.02 g/l TOC	Lyoph., reconst. to 100×	N.D. [e]	N.D. [e]	N.D. [e]

Chlorination was performed using a 1 : 1 ratio of chlorine equivalents per mole of carbon. Mutagenesis assays were carried out in duplicate, without the addition of S9.

Negative control values (mean total revertants/plate) were: TA98 = 56, TA100 = 128.

Positive control values (mean net revertants/plate) were: 1 μg of 2-nitrofluorene (TA98) = 176; 1 μg of Na azide (TA100) = 1021.

[a] numbers in parentheses indicate % recovery of TOX.

[b] numbers in parentheses indicate % recovery of mutagenic activity.
Recoveries are based upon levels in the 1 g/l TOC sample before lyophilization.

[c] before lyophilization.

[d] after lyophilization.

[e] Not detectable.

Fig. 5 shows the effect of varying the chlorine concentration during chlorination at neutral pH. The humic concentration was standardized at 1.0 g/l TOC for each chlorine concentration used. Mutagen formation and TOX formation appeared to be linearly related to amount of chlorine added over the concentration range tested. Significant mutagenicity could be detected with as low as 0.3 : 1 chlorine to carbon. Chlorine concentration above 1 chlorine equivalent per mole of carbon resulted in the presence of a free chlorine residual greater than 10 mg/l, which was found to be highly toxic to the tester bacteria as evidenced by a substantial clearing of the background bacterial lawn. Removal of the chlorine residual by titration with sodium thiosulfate (data not shown) revealed that mutagen production was still increasing with chlorine to carbon ratios up to 2 : 1, but no longer in a linear fashion.

Correlation coefficients between TOX levels and mutagenic activity levels were calculated from the data in Figs. 3 and 5. These were found to be 0.94 for TA98 activity and TOX, and 0.81 for TA100 activity and TOX, indicating a high

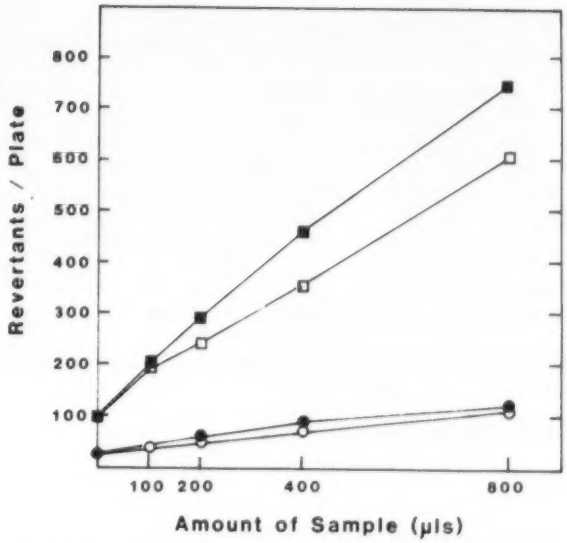

Fig. 4. Effect of purging on mutagenic activity of chlorinated humic acids. Samples were prepared at 1 g/l TOC, chlorinated at a 1:1 Cl:C ratio at pH 7 without buffer and then purged for 2 h at 23°C with helium. ●, TA98 mutagenesis before purging; ○, TA98 mutagenesis after purging; ■, TA100 mutagenesis before purging; □, TA100 mutagenesis after purging.

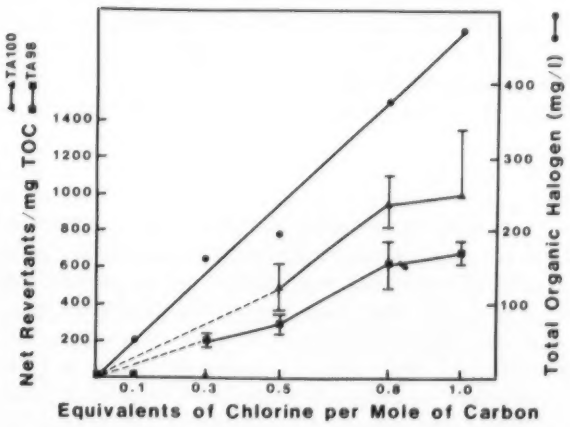

Fig. 5. Effect of varying the chlorine concentration during humic chlorination on mutagen formation and organic halogen formation. Mutagenicity values are slopes ± standard errors of dose–response curves at each chlorine concentration.

TABLE 2

EFFECT OF CHLORINATION pH ON MUTAGEN FORMATION AND ORGANIC HALOGEN FORMATION

Pre-chlorination pH	Post-chlorination pH	TOX [a] (mg/l)	Net revertants/ml ± S.E.	
			TA98	TA100
7.0	2.75 ± 0.25	445 ± 43	517 ± 35	2025 ± 244
6.8 [b]	6.4 ± 0.05	297 ± 48	107 ± 20	243 ± 65
11.5	7.6 ± 1.0	217 [c]	N.D. [d]	195 ± 17

pH and TOX data are means ± SD from 2 Expts.; mutagenicity data are mean slope values ± S.E. of slopes.

Control values were: distilled H_2O, TA98 = 14 ± 6, TA100 = 116 ± 16; 2-nitrofluorene (1 μg) TA98 = 270 ± 62; Na azide (1 μg) TA100 = 805 ± 116.

[a] Total organic halogen.

[b] Potassium phosphate buffer was added to prevent drop in pH.

[c] Data from 1 Expt. only.

[d] Not detectable.

correlation between organic halogen formation and mutagen formation.

Based on the results shown in Figs. 3 and 5, the reaction conditions were standardized at 1 g/l TOC humic concentration and a 1 : 1 ratio of Cl : C. Using these conditions, the effect of varying the reaction pH was examined (Table 2). When chlorination was carried out without the addition of buffer, a pH drop of about 4 units was observed regardless of whether the initial pH was neutral or highly basic. The addition of potassium phosphate buffer to the reaction at neutral pH effectively prevented the pH drop and resulted in an 80–90% reduction in mutagen formation as compared to the unbuffered reaction at neutral pH. The level of TOX formed was also lower under the buffered reaction conditions, but only by about 30%. No TA98 mutagenic activity was detected in the humic samples which were chlorinated at high pH. TA100 mutagenesis at high chlorination pH was 90% lower and TOX formation was about 50% lower than in the neutral pH, unbuffered reaction.

Stability of the formed mutagens

One explanation for the reduced levels of mutagens at higher reaction pH might be that mutagens are formed but are rapidly degraded to inactive compounds due to the alkali lability of the mutagens. Experiments were carried out to explore this possibility. The results in Table 3 show that mutagenic activity was rapidly lost upon raising the pH of a chlorinated humic sample from 3 to 11.5. After 1 h less than 20% of the activity remained and within 24 h activity was no longer detectable at 23°C storage temperature. On the other hand, raising the pH from 3 to 7 resulted in a loss of only 54% (TA98) to 67% (TA100) of the activity after storage for 4 days at 23°C. In both cases the loss of activity was found to be irreversible, since no activity was regained upon lowering the pH back to 2.8 (data not shown). Maintenance of the samples at 4°C effectively prevented the degradation of the mutagens at pH 7. It

TABLE 3

pH STABILITY OF MUTAGENIC ACTIVITY AS A FUNCTION OF TIME AND TEMPERATURE

pH [b]	Temp. (°C)	Net revertants/ml ± S.E. [a]									
		1 h		4 h		24 h		4 days		7 days	
		TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100
2.8	4°	383 ± 12	2303 ± 98	335 ± 23	2317 ± 167	291 ± 27	1943 ± 78	291 ± 27	2067 ± 74	216 ± 18	2226 ± 105
2.8	23°	–	–	350 ± 20	2270 ± 81	398 ± 40	1978 ± 48	307 ± 17	2071 ± 95	160 ± 24	1872 ± 31
7.0	4°	313 ± 26	1708 ± 64	232 ± 74	1752 ± 74	300 ± 27	1878 ± 76	292 ± 16	1547 ± 91	158 ± 12	1622 ± 28
7.0	23°	305 ± 22	1631 ± 11	190 ± 14	1580 ± 69	230 ± 26	1142 ± 62	135 ± 14	683 ± 70	70 ± 23	572 ± 32
11.5	4°	47 ± 9	409 ± 21	57 ± 8	303 ± 36	50 ± 10	347 ± 39	24 ± 5	107 ± 14	N.D.	N.D.
11.5	23°	58 ± 8	297 ± 38	35 ± 16	252 ± 22	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D., not detectable.

Mean values ± SD positive and negative controls were as follows: dist. H_2O control, TA98 = 22 ± 6.5, TA100 = 141 ± 51; pos. control (spontaneous subtracted) for TA98, 2 μg 2-nitrofluorene = 289 ± 73; for TA100 1 μg sodium azide = 750 ± 141.

[a] All values are from assays performed with triplicate platings at 3 dose levels without the addition of S9, and represent combined data from 2 Expts.

[b] Preparation and pH adjustment of samples is described in Materials and Methods. The buffering capacity of the 0.1 M sodium phosphate (pH 7.4), which is a routinely added to the top agar, prevented the actual assay pH from going below 7.25 or above 7.65.

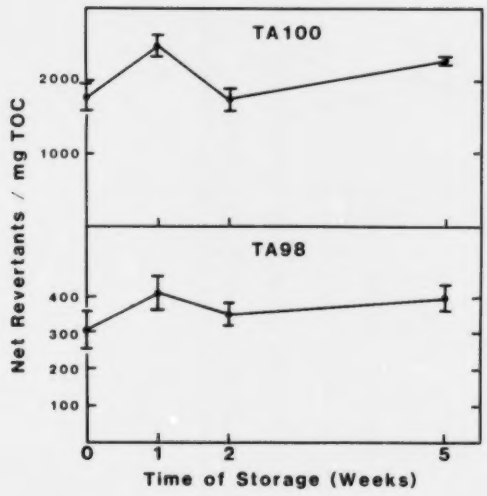

Fig. 6. Stability of mutagenic activity of a chlorinated humic sample with duration of storage at 4°C. Conditions for sample preparation and chlorination are the same as for Fig. 4, except no purging was performed.

should be noted that after the initial (0 time) pH adjustments a decline in pH was observed, which continued throughout the experiment. This required readjustment of pH at each sampling time in order to maintain the pH between 6.0–7.0 and 10.5–11.5, respectively.

In a separate experiment the stability of the mutagens was examined with respect to duration of sample storage at acid pH for up to 5 weeks. Fig. 6 shows that mutagen levels, in both strains TA98 and TA100, remained essentially unchanged when stored at pH 2.2 at 4°C in the dark.

Discussion

The work of Loper et al. (1978), Glatz et al. (1978), Nestmann et al. (1979), Forster et al. (1981), Heartlein et al. (1981), and Kool et al. (1981) has demonstrated that mutagenic chemicals commonly occur in finished drinking water. There are 3 general sources from which these chemicals might arise: (1) natural products in source waters; (2) industrial/agricultural contamination of source waters; (3) products arising during water treatment and/or distribution. There have been data which suggest that the use of chlorine as a disinfectant may be at least partially responsible for the presence of non-volatile mutagens in drinking water (Cheh et al., 1980; Maruoka and Yamanaka, 1980; de Greef et al., 1980). However, the potential substrates for the formation of mutagenic substrates in drinking water have not received systematic study.

Humic substances are likely candidates as precursors to mutagen formation since they account for a major portion of the organic matter in most surface waters, and are known to be precursors of trihalomethanes as well as other volatile and non-volatile halogenated organics which are formed during water chlorination (Stevens et al., 1978; Rook et al., 1976; Oliver, 1978, Christman et al., 1980). Furthermore, recent work from this laboratory (Bull et al., 1982) has shown that aquatic humic and fulvic acids react with chlorine to produce direct-acting mutagens. The present work confirms these preliminary observations and further examines the properties of the observed mutagens as well as the reaction conditions responsible for their formation.

Following chlorination of humic acid at neutral pH, direct-acting mutagenic activity was observed for all 5 of the standard Salmonella tester strains, indicating the presence of compounds which cause both base-pair substitution mutations (TA1535 and TA100), and frameshift mutations (TA1537, TA1538 and TA98). Previously, positive mutagenic responses were observed only for TA98 and TA100 (Bull et al., 1982). This discrepancy is most likely attributable to the fact that the assay detection capabilities were increased in the present study by using a slightly higher chlorine to carbon ratio during chlorination (1 : 1 vs. 0.8 : 1), and by increasing the maximum amount of sample added to the assay plates by 2-fold. The addition of rat liver microsomal enzymes (S9 fraction) plus NADPH-generating cofactors to the mutagenesis assay resulted in a substantial inactivation of mutagenic compounds, the extent of which was strain-specific. This inactivation is not simply a result of non-specific protein binding because the addition of bovine serum albumin did not affect the degree of inactivation by S9, nor did BSA alone result in inactivation of the mutagens.

The advantage of carrying out the chlorination reaction at high substrate levels was that concentration of sample was not needed. This avoided problems with potential artifacts and/or selectivity in recovery of compounds that occur with all methods of sample concentration (Jolley, 1981). Conversely, there was some question as to whether mutagen production was attributable to the use of such high substrate concentrations. This was found not to be the case since similar specific mutagenic activities (revertants/mg TOC) were obtained even at 10-fold lower humic concentrations, as is evident from the linearity of mutagenic response with respect to humic concentration (Fig. 3). Furthermore, at a humic acid TOC level of 22 mg/l, chlorination also resulted in mutagen formation, although detection of mutagenic activity required concentration of the sample. This level of humic acid is still about 5–10-fold higher than the TOC levels (comprised mainly of humic material) of surface waters in most U.S. cities (Symons et al., 1975), but it approaches the TOC levels in several highly colored water sources in the southern United States and in some sources charged by wood pulp industries. The level of mutagens formed at these more realistic humic concentrations appeared to be lower, after correction for difference in initial organic content, than at the higher concentrations. However, the interpretation of results was complicated by concentration dependent effects on recovery efficiencies of both mutagen and organic halogen content of samples (Table 1). In fact, when the mutagenic activities were calculated on the basis of

organic halogen content the activities were found to be very similar. It is also important to note that the amount of TOX formed per mg of initial TOC was nearly the same for both the 22 mg/l and 1 g/l TOC samples (before lyophilization) with levels of 0.11 and 0.13 moles TOCl chlorine per mole TOC carbon, respectively. This indicates that approximately the same degree of chlorine substitution is occurring over this range of humic concentration, and suggests that similar reaction products are being formed.

The major portion (80–90%) of the mutagenic activity formed after chlorination was still present following lyophilization (Table 1) or purging (Fig. 4) of the samples. The more volatile compounds present would be expected to be removed by such treatments. These results support the conclusion that non-volatile compounds are primarily responsible for the mutagenicity found after chlorination of humic acid.

The pH at which the chlorination reaction was carried out was found to be a critical factor in mutagen formation. The highest levels of mutagens were observed after chlorination at neutral pH without buffer during which the pH drifted downward to a final pH between 2 and 3. Mutagen levels were reduced substantially when the pH was maintained at 7 by the addition of buffer, indicating that maximum mutagen formation occurred at pH below 7. Furthermore, there was no evidence of mutagen formation after chlorination at pH 11.5. A pH dependency of mutagen formation is consistent with the observations of Oliver (1978) who found that non-volatile chlorinated organic carbon formation following chlorination of humic acid decreased with increasing pH. This is directly opposite the pattern for the formation of volatile halogenated organics, including the trihalomethanes, which occurs greatest at high pH (Stevens, 1979; Oliver, 1978).

An alternative explanation to decreased formation of mutagens at pH 7 and above is that the rate of mutagen formation is slower, or that the mutagens are less stable at the higher pHs. Mutagen instability was observed upon raising the sample pH and was found to be dependent upon both incubation time and temperature (Table 3). Certainly at a pH of 11.5 the rapid degradation of mutagenic activity, regardless of temperature, could explain the near absence of detectable activity upon chlorination at this pH. On the other hand, at pH 7 degradation of activity was much slower and highly temperature dependent. The extent of degradation which occurred after 4 days at 23°C could not totally account for the 80–90% lower activity observed upon chlorination at this pH under similar incubation conditions (Table 2).

Identification of the chemicals responsible for the mutagenic activity is the subject of continued study. However, studies on chlorination stage effluents from pulp and paper mills should provide some insight into the identity of the mutagens. Nazar and Rapson (1980) have shown that the reaction of chlorine with the lignin portion of wood pulp is responsible for mutagen formation in these effluents. Because lignin is thought to be one of the major precursors in humic and fulvic acid formation, and because many functional groups are shared by humic and lignitic structures (Schnitzer and Khan, 1972), it seems likely that mutagenic products from the reaction of chlorine with these 2 polymeric compounds would be similar. Based on a substantial phenolic content of the polymers, Nazar et al. (1981) have proposed

the use of catechol as a model substrate for reaction with chlorine, and have identified *o*-benzoquinone and chloro-*o*-benzoquinone as major mutagenic reaction products. On the other hand, Kringstad et al. (1981) have attributed significant mutagenic activity to the presence of 1,3-dichloroacetone and 2-chloropropenal in kraft pulp bleaching effluents. A number of other compounds, including chlorinated alkanes and alkenes, and chlorinated acetones have also been identified in chlorination stage pulp mill effluents and found to possess mutagenic activity (Nestmann et al., 1980; McKague et al., 1981; Douglas et al., 1982).

One common feature of these mutagenic constituents of pulp mill chlorination stage effluents is their alkali lability (McKague et al., 1981; Rapson et al., 1980; Nazar et al., 1981; Douglas et al., 1980). Nazar and Rapson (1982) have recently shown that the decay of mutagenicity at high pH is accompanied by cleavage of organically bound chlorine by hydroxide ion. This is strong evidence that chlorine substitution products are primarily responsible for the mutagenic activity. The results from the present study are consistent with chlorine substitution as also being the primary reaction mechanism involved in mutagen formation during chlorination of humic acid. The mutagenic activity also demonstrates alkali lability accompanied by consumption of hydroxyl ion, (Table 3), and shares other properties with wood pulp chlorination products. In both cases, the mutagenic compounds produced do not require metabolic activation to demontrate mutagenic activity and in fact are inactivated by rat-liver S9 fractions, and they are highly stable upon storage at acid pH (Fig. 6; Eriksson et al., 1979). Furthermore, organic halogen formation closely paralleled mutagen formation (Figs. 3 and 5). The level of chlorine incorporation into the humic acid molecule was substantial, amounting to an average of one chlorine atom per 10 carbon atoms after chlorination at a 0.8 : 1 ratio of chlorine equivalents per mole of carbon. The high correlation between TOX levels and mutagen levels does not, in itself, establish a cause–effect relationship. Undoubtedly, oxidation reactions are also occurring under these chlorination conditions, which may also result in humic oxidation products being formed at rates which correspond to mutagen formation.

The mutagens detected in the chlorinated humic solutions also have properties similar to those often found in drinking water. Drinking water mutagens are usually direct-acting, non-volatile compounds which are decreased in activity by the addition of S9, and they are most efficiently detected using strains TA98 and TA100 (Loper, 1980a). Alkali sensitivity has also been observed for mutagenic activity in drinking water (Loper, 1980b).

At present it appears that the use of commercial humic acid as a model substrate is appropriate for examining the formation of mutagenic chemicals during water treatment. It allows both the chemical and biological properties of disinfection reaction products to be studied under controlled conditions, a situation that is difficult to achieve under ambient conditions. Furthermore, the influence of variables such as humic concentrations, chlorine concentration, pH, etc. can be individually evaluated. Confirmation of the validity of this model, however, requires identification of the mutagenic compounds and verification of their occurrence in drinking water.

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